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13. ABSTRACT (Maximum 200 Words)

Inactivation of *int6* has been linked to breast cancer formation, but its molecular function and precise role in tumorigenesis are largely unknown. This project tests the hypothesis that *int6* is a tumor suppressor gene, regulating the proteasome to mediate genetic stability and cell division. The specific aims of this proposal are to determine whether disrupting Int6 function can influence (1) proteasome functioning and (2) chromosome segregation in human cells. I have generated siRNAs that successfully knock-down Int6 expression in HeLa and human mammary epithelial cells, and HeLa cells with reduced Int6 expression display mitotic defects. The next step is to generate stable human mammary cells with reduced Int6 level to carry out detailed studies on chromosomal segregation and proteasome functioning. In addition, I engaged in mechanistic study in *S. pombe* to better understand the regulation of the proteasome by Int6. Like many proteasome subunits, Int6 contains a PCI domain, and it may play an important role in influencing tumorigenesis. I have identified an essential residue in the PCI domain, and have validated its importance in Int6 and a proteasome subunit Rpn7. Furthermore, my data suggests that nuclear import of proteasome subunits might involve an importinα-independent mechanism, specifically mediated by the importin β Kap123. In the future, I will next examine whether Int6 regulates Kap123 in both yeast and humans.

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Introduction

"*int6*" gene is a locus where Mouse Mammary Tumor Virus (MMTV) insertion leads to tumor formation [1]. It was also shown to be important for breast tumorigenesis in humans [2-4]. However, its molecular function is poorly understood. Using the fission yeast *Schizosaccharomyces pombe* as a model system, we discovered a conserved function of Int6, which is to regulate the nuclear import, assembly, and proper functioning of the 26S proteasome [5-7]. These findings suggest that disruption of Int6 might lead to breast tumorigenesis in human by weakening the 26S proteasome, leading to accumulation of regulatory proteins, some of which are essential for proper chromosome segregation and cell cycle progression. Thus, **this project investigates the hypothesis that a key function of Int6 is to control proteasome function, and this function is conserved in human to influence breast tumor formation.** The specific aims of this proposal are to determine whether disrupting Int6 function can influence (1) proteasome functioning and (2) chromosome segregation in human mammary epithelial cells.

Report Body

Task 1: Generate cell lines with reduced Int6 levels

Progress:

a. Generation of anti-Int6 poly-clonal antibody

I propose to generate siRNA that can knock down Int6 expression, the detection of which requires an anti-Int6 antibody. I have successfully raised a poly-clonal Int6 antibody, which efficiently recognizes endogenous and ectopically expressed human Int6 in immortalized but non-cancerous human mammary epithelial MCF10A cells (Figure 1A). This antibody is now named Int6-N1.

b. and c. Knock-down of Int6 in by siRNA

I have designed and tested several siRNAs to knock down Int6 expression by direct transfection. While we are testing these synthetic siRNAs, Morris and Jalinot reported successful knock down of Int6 expression in HeLa cells using two siRNA sequences (I6.1. and I6.3.) [8]. My results indicate that the siRNA designed and synthesized by us (IN550) can also efficiently knock down Int6 in HeLa cells (Figure 2A and B). Thus, we have so far collected at least three siRNAs to efficiently knockdown Int6.

Next, I further examined whether my siRNA can also knock down Int6 in human mammary epithelial MCF10A cells. My preliminary data shows that this siRNA can also successfully knock down Int6 in MCF10A cells, although the efficiency is not as high as in HeLa cells (Figure 2C). This difference in knock-down efficiency may be due to difference in transfection efficiency or expression regulation, which will be examined next to achieve more efficient knock-down of Int6 in MCF10A cells.

Task 2: Analyze genetic stability and mitotic abnormalities in engineered cell lines

Progress:

We have established live cell DNA staining protocols to investigate mitotic phenotypes. From initial analysis of HeLa cells with reduced Int6 expression as obtained in the previous task, I have observed multi-nuclear cells using live cell DNA staining, indicating potential mitotic defects (Figure 2D). In order to carry out detail mitotic study and long-term genetic stability analysis, I will need to build stable human mammary

epithelial cell lines with reduced Int6 expression. I am currently in the progress of building expression vectors for siRNA to achieve this goal.

Task 3: Analyze proteasome functions in engineered cell lines

Progress:

I propose to investigate whether Int6 physically associates with the proteasome in human mammary epithelial cells by immunoprecipitation. For this purpose, I am currently establishing the co-immunoprecipitation protocol to determine whether proteasome subunits can be pulled down together with Int6. As shown in Figure 1, we have raised a good anti-Int6 polyclonal antibody, and I will further test its ability to immunoprecipitate Int6 from MCF10A cells. As an alternative, my colleague has established a stable MCF10A cell line expressing the cmyc-tagged Int6, in which Int6 can be pulled down by anti-cmyc antibody (Figure 1A). I am also collecting and generating antibodies against proteasome subunits. The Rechsteiner lab has kindly provided us with antibodies that recognize many proteasome subunits (10 in total) [9]. Among all the proteasome subunits, Rpn5 is best characterized in our fission yeast model system to physically interact with Int6, and to be directly regulated by Int6. Thus, Rpn5 will be among the proteasome subunits to test for binding with Int6 in human cells. Unfortunately, anti-Rpn5 antibody, to our knowledge, is not available. Therefore, I am in the process of generating my own anti-Rpn5 polyclonal antibody. Last year, I generated one Rpn5 polyclonal antibody by using a peptide corresponding to the N-terminus of Rpn5 as antigen, and this antibody can recognize Rpn5 when it is expressed in *S. pombe* (Figure 1B). However, the antibody has a high background, and the titer appears to be low. I will further test its efficiency to recognize Rpn5 when expressed in MCF10A cells. In the mean time, I am using bacterial expressed and purified GST-Rpn5 protein to generate better anti-Rpn5 antibodies.

The other part of this task is to investigate whether human mammary epithelial cells with reduced Int6 level will be deficient in proteasome functioning. Detailed studies will be carried out once human mammary epithelial cells with stably reduced Int6 expression are obtained. In the mean time, I am planning to examine transiently transfected HeLa cells and MCF10A cells mentioned in Task 1 to determine sensitivity to proteasome inhibitor MG132.

Additional Progress:

In addition to the proposed experiments, I have explored other areas in the study of Int6:

Structure-Function analyses of Int6:

MMTV insertion into mouse *int6* locus generates C-terminally truncated Int6ΔC, and expression of these truncated proteins can transform human mammary cells. The truncated Int6 proteins lack the PCI domain, which is also found in many proteasome subunits, including Rpn5 and Rpn7, suggesting that the PCI domain may play a critical role in interacting with the proteasome. When using the Int6-N1 antibody to investigate potential changes in Int6 proteins in human breast cancer cell lines, my colleagues found evidence that Int6 truncation in the C-terminus, potentially causing the loss of the PCI domain, may occur frequently in human breast cancer cell lines. In complement to these studies, I carried out structure-function analyses of the PCI domain to define its molecular functions: First of all, I mutated all the conserved residues in the PCI domain of *int6*, and tested them functionally in *spint6* null *S. pombe* cells. Among all these mutations, only the change of Leu332 to Asp abolished the ability of spInt6 to rescue the growth and proteasome defects of *spint6* null *S. pombe* cells (Figure 3A and B), and the mutant human Int6 is similarly inactive in yeast.

To determine whether this Leucine represents an essential residue for all the PCI proteins, I next examined whether this residue is also important for at least two additional proteins, Rpn7 and Rpn5. I found that Rpn7L289D is also unable to bind other proteasome subunits and is mislocalized in *S. pombe* cells (Figure 4). Mutagenesis experiment on Rpn5 is currently in progress. As I am carrying out these structure-function analyses, our collaborators used a bio-informatic approach and suggested that the hydrophobicity of this Leucine might be important for maintaining the structure of the PCI domain.

These studies suggest that the PCI domain is essential for proper proteasome functioning, supporting the hypothesis that *int6* influence tumorigenesis by regulating the proteasome. To follow up on these studies in humans, I will investigate whether Int6 carrying the same mutation will be deficient in binding the proteasome, and whether Rpn7 and Rpn5 carrying the same mutation will mislocalize in human cells.

Determining the specific importin(s) responsible for proteasome import:

Proteasomes enter the nucleus in both human[10] and in *S. pombe*, and our model predicts that Int6 regulates proteasome nuclear import and assembly. To better understand how *int6* regulates proteasome import, I have examined the cellular import machinery responsible for import of proteasome subunits, and whether *int6* plays a role in regulating this process. How proteasome subunits enter the nucleus is poorly understood. For example, although proteasome concentrates inside the nucleus, many proteasome subunits do not have a classic nuclear localization sequence (NLS). Protein nuclear import is mediated by the importin α and β proteins. I first used the *S. pombe* system, in which mutants of all importin α and β are available. Using the GFP-Rpn7 as a reporter, I found that it is mislocalized specifically in an importin β mutant, the *kap123null* mutant (Figure 5 and Table 1). Previous studies suggest that this importin β might mediate nuclear import without importin α [11, 12], and my results confirmed this by showing that classical NLS-mediated import appears normal in *kap123null* cells. These initial investigations support an idea that an importin α-independent nuclear import machinery specifically mediated by Kap123 might be responsible for proper nuclear import of proteasome subunits, which may explain why non-NLS proteasome subunits can enter the nucleus. In the future, I will determine whether Int6 regulates this protein in both *S. pombe* and humans.

Key Research Accomplishments

1. I have generated and confirmed anti-human Int6 antibody in human mammary epithelial cells (Figure 1).

2. I have generated siRNA sequence that can transiently knock-down Int6 expression in both HeLa cells and MCF10A cells by direct transfection (Figure 2).

3. I have identified a conserved leucine residue in the PCI domain, which is essential for proper localization, assembly and functioning of both Yin6 and proteasome subunit Rpn7 in fission yeast (Figure 3 and 4).

4. I have evidence suggesting that a Kap123-mediated, but importin α-independent nuclear import mechanism might be responsible for nuclear import of proteasome subunit (Figure 5).

Reportable Outcomes

1. **Polyclonal anti-Int6 antibody Int6-N1.**

2. **siRNA sequence (IN550) for reducing Int6 expression in human mammary epithelial MCF10A cells.**

3. **Posters:**

3.1. Sha Z., Cabrera R., and Chang EC., Regulation of proteasome nuclear import and assembly by Int6/Yin6. The 26th Annual Department of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. Houston, TX. April, 2004.
3.2. Sha Z., Cabrera R., and Chang EC., Regulation of proteasome nuclear import and assembly by Int6/Yin6. The 2004 ASBMB/IUBMB Annual Conference. Boston, MA. June, 2004.
3.3. Sha Z. and Chang EC., Regulation of proteasome nuclear import and assembly by Int6/Yin6. 16th Annual Graduate Student Research Symposium, Baylor College of Medicine. Houston, TX. October, 2004.

4. **Oral Presentations:**

4.1. Regulation of proteasome nuclear import and assembly by Int6/Yin6. Baylor College of Medicine Breast Center Data Review. Houston, TX. May, 2004.
4.2. Regulation of proteasome nuclear import and assembly by Int6/Yin6, Baylor College of Medicine Breast Center Data Review. Houston, TX. Jan, 2005
4.3. Regulation of proteasome nuclear transport and assembly by Int6/Yin6. Society of Chinese BioScientists in America annual symposium. Houston, TX. April, 2005.

Conclusions

During the first year, I have accomplished a major objective, that is to reduce Int6 expression in human mammary epithelial cells by siRNA. Furthermore, using these cells with reduced Int6 expression, I observed multi-nuclei cells in HeLa cells, validating our hypothesis that Int6 inactivation leads to mitotic defects. The next step is to construct stable cell lines with reduced Int6 expression, which is required to carry out more detailed assays for chromosome segregation, genetic stability, and proteasome functioning.

To complement my studies with mammalian cells, I am also using the model system *S. pombe* to better study the mechanism by which proteasome import and functioning are regulated by Int6. Progress has been made in two main areas. First, I have identified a potentially essential residue for the PCI domain and validated its importance for at least two PCI proteins, Int6 and Rpn7. In the future, I will investigate whether this residue is important for Int6 and proteasome functioning in humans and examine human breast cancer samples to see if this region is frequently mutated. Second, I found that proteasome nuclear import might specifically require an importin β kap123, but might not require importin α, and in the future I will examine whether Int6 regulates Kap123 in both yeast and human.

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Appendices

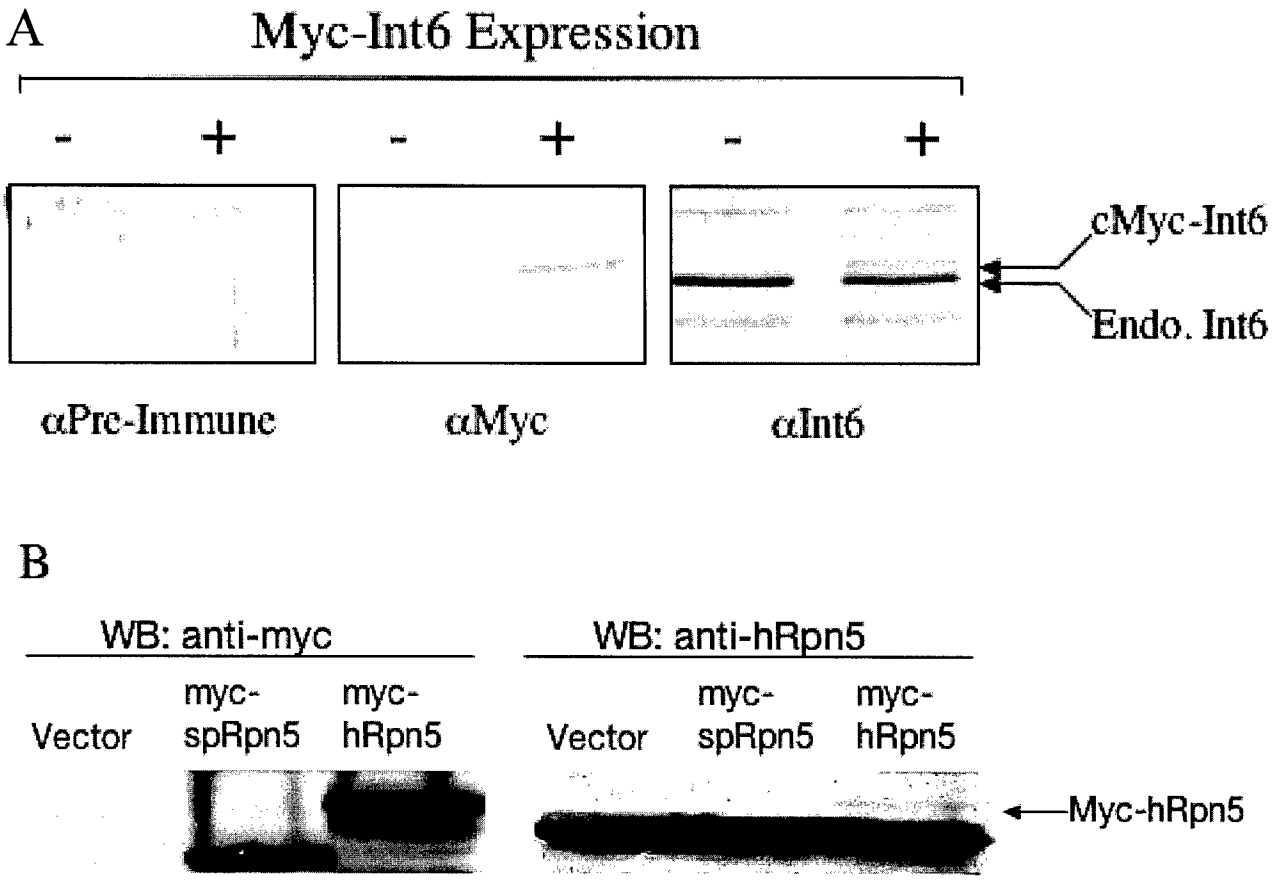


Figure 1. Testing of anti-Int 6 and anti-Rpn5 antibody

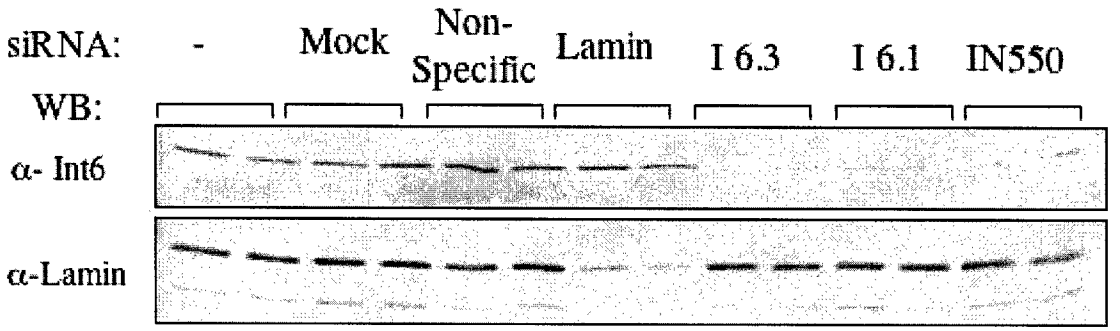
A. Detection of Int6 in MCF10A cells by anti-Int6 antibody Int6-N1. MCF10A cells are tranfected by a vector expressing Myc-Int6 or by an empty vector. Cell lysate was subject to Western blotting with α-Myc antibody or α-Int6 antibody Int6-N1. Int6-N1 recognizes both the endogenous Int6 and the ectopically expressed Myc-Int6.

B. Detection of Rpn5 in *S. pombe* by anti-Rpn5 antibody. Myc-tagged human Rpn5 (myc-hRpn5) or *S. pombe* Rpn5 (myc-spRpn5) are ectopically expressed in *S. pombe* cells, and cell lysate was subject to Western blotting with α-Myc antibody or α-Rpn5 antibody. My anti-Rpn5 antibody recognizes only ectopically expressed myc-hRpn5, but cannot cross-react with *S. pombe* Rpn5.

A

IN550 GCAGCCAUGGAAGACCUUACA 570
547 TACGUCGGUACCUUCUGGAAU

B



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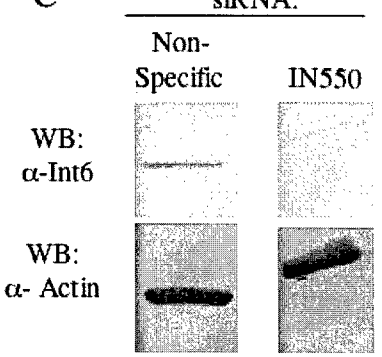

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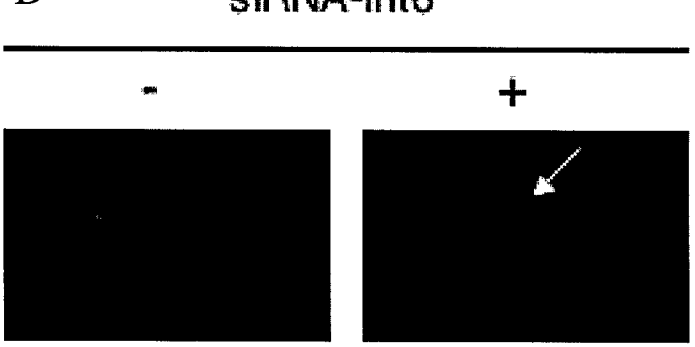

Figure 2: Suppression of Int6 levels in HeLa Cells and MCF10A Cells by synthetic siRNA

(A) Sequence of siRNA (I550) designed for suppressing Int6 protein levels in human mammary epithelial cells.

(B) Anti-Int6 western blotting (top) to confirm successful suppression of Int6 level in HeLa cells transiently transfected with indicated siRNA. Non-specific siRNA and anti-Lamin siRNA were included as negative and positive controls, respectively, and anti-lamin (bottom) western blotting was included as loading control and to confirm the effect of the siRNA-mediated suppression of gene expression. Cell lysate was prepared in duplication.

(C) Anti-Int6 western blotting to confirm successful suppression of Int6 level in MCF10A cells transiently transfected with indicated siRNA. β-actin was included as loading control.

(D) Abnormal mitotic cells when Int6 expression is knocked down. HeLa cells in which Int6 expression has been reduced by siRNA were stained to reveal DNA, and cells with three nuclei (arrow) can be readily detected

A spRpn7L289/spInt6L332

```
hsRpn7    266 CRYSVFFQSLAVVEQ.EMKKDWLFAPHYRYYVREMRIHAYSQLLESYRSL 314
mmRpn7    266 CRYSVFFQSLAIVEQ.EMKKDWLFAPHYRYYVREMRIHAYSQLLESYRSL 314
spRpn7    280 CDYSGFFRTLADVEVNHLKCDQFLVAHYRYYVREMRRRAYAQLLESYRAL 329
hsINT6    303 FDFDGAQKKLRECES.VLVNDFFLVACLEDFIENARLFIFETFCRIHQCI 351
mmINT6    303 FDFDGAQKKLRECES.VLVNDFFLVACLEDFIENARLFIFETFCRIHQCI 351
spINT6    323 VDFEKAQHCLRECEE.VLKTDFFLVSLCDHFLEGARKLLAEAYCRIHSVI 371
spRpn5    306 PKIAEIYGSLLRSNPVFAENDEKGEKRWSELRKRVIEHNIRVVANYYSRI 355
spRpn3    333 GDLCAFTDALSKYEAEFRFDG..LYTLICRLRHTVIKTGLRMISLSYSRI 380
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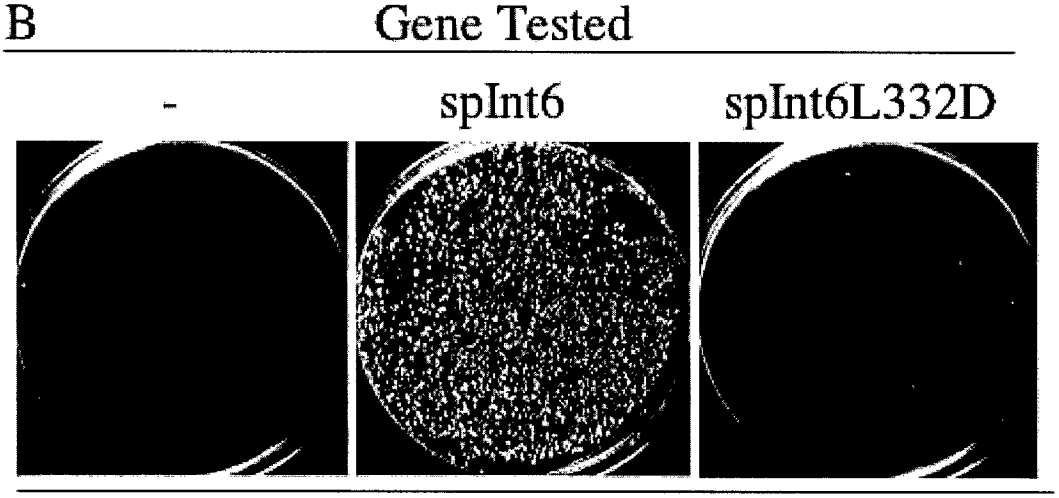
B



Growth of *spint6* null cells at 20°C

C

Relevent Genotype	% survival in Canavanine (12 μg/ml)
WT	102.3%
yin6Δ	24.3%
yin6 L332D	23.5%

Figure 3: Leucine 332 of spInt6 is essential for its proper functioning

(A) Sequence allignment showing the conserved leucine residue among several PCI proteins including Int6 and Rpn7.

(B) Leu332 in the PCI domain of spInt6 is critical for its function. *spint6* null cells transformed with a vector control or the same vector carrying wild-type or mutated *yin6* were spread on plates and incubated at 20°C for 6 days. spInt6, whose Leu332 is mutated to Asp, loses ability to rescue cold-sensitive growth defect of *spint6* null cells.

(C) The PCI domain mutated spint6 strain is sensitive to the arginine analog, canavanine, to which proteasome mutants are similarly sensitive. Equal numbers of cells were spread on plates with or without canavanine sulfate (12 μg/ml) and incubated at 30°C for 14 days. The number of colonies that emerged on plates without canavanine was taken as 100% survival.

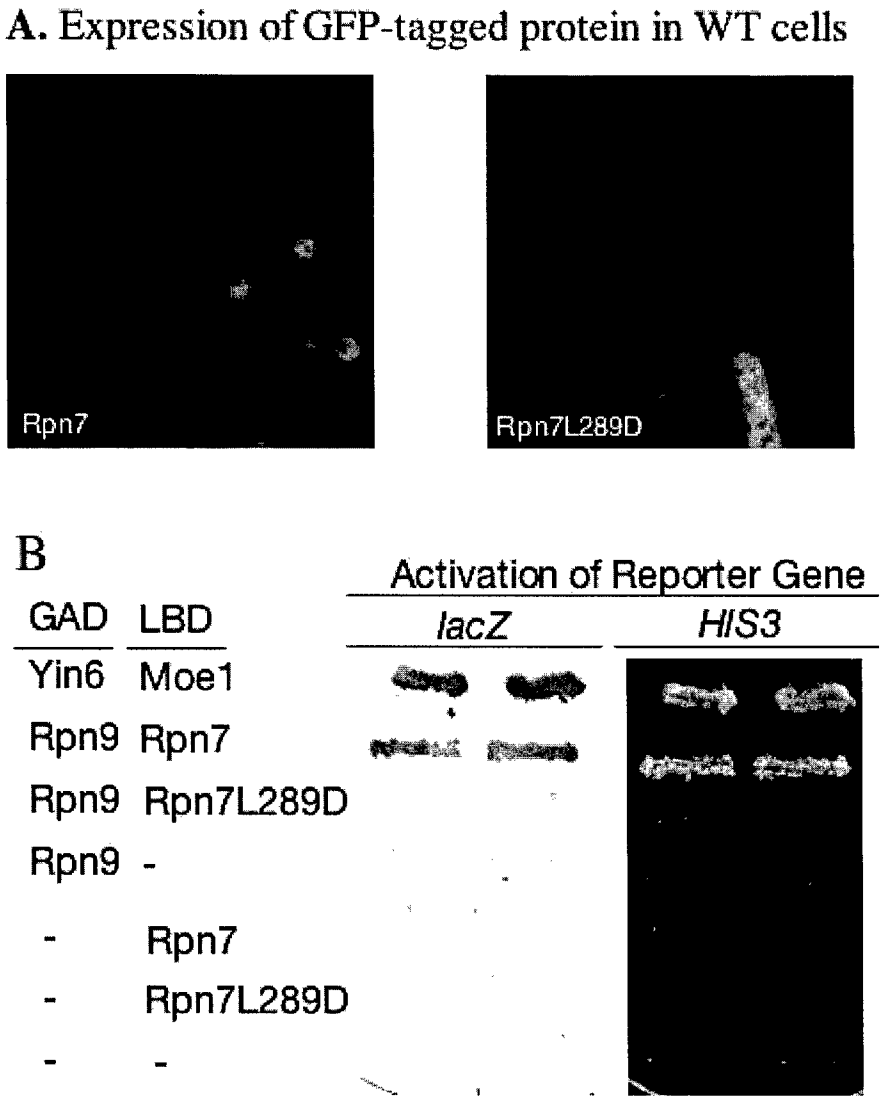


Figure 4 Rpn7L289D is deficient in binding Rpn9, and mis-localizes in *S. pombe* cells

(A) Mislocalization of Rpn7L289D in *S. pombe* cells. GFP-tagged wild type or mutant Rpn7 was expressed in wild type *S. pombe* cells, and localization was examined by fluorescent microscopy. Expression levels of wild type and mutant protein are similar (data not shown). While GFP-Rpn7 shows clear nuclear localization, specifically concentrated at the nuclear rim (left panel), GFP-Rpn7L289D is completely diffused in the cell (right panel).

(B) Deficiency of Rpn7L289D in binding a proteasome subunit, Rpn9, as measured by the Yeast Two-Hybrid assay. Proteins fused with the LexA DNA binding domain (LBD) and Gal4 activation domain (GAD) are as indicated. *lacZ* and *HIS3* are the reporter genes in this experiment.

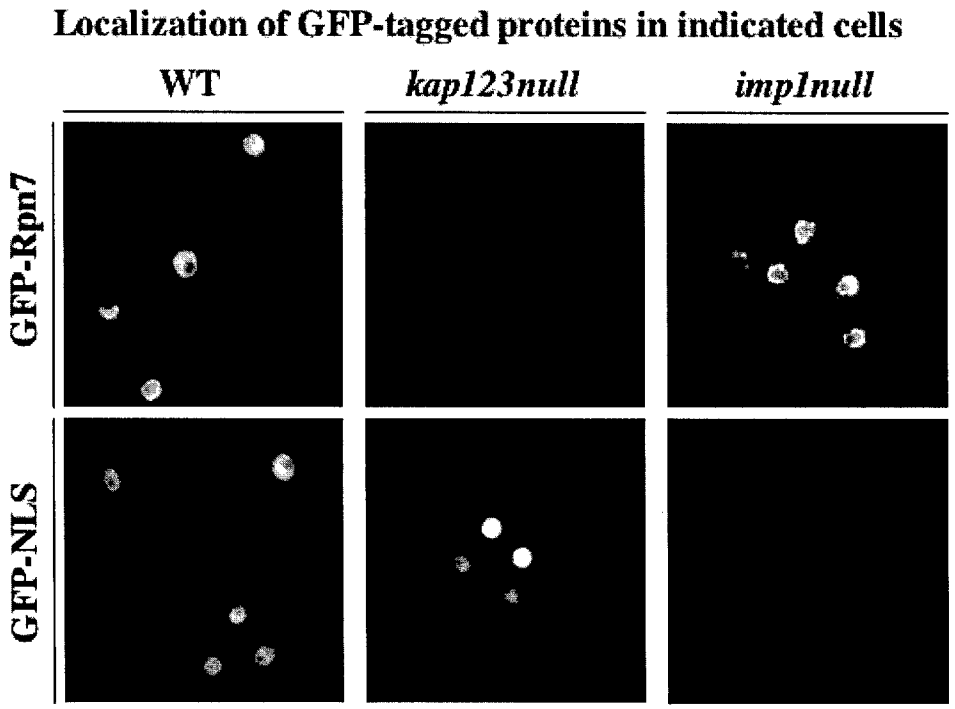


Figure 5. Mislocalization of GFP-Rpn7 in *kap123null* cells
GFP-Rpn7 was expressed in wild-type and mutant *S. pombe* cells at 25°C overnight, and localization was examined by fluorescent microscopy. A GFP-NLS construct is included as control, and is mis-localized in importin α deficient cells (*imp1null*). Notice the diffused localization of GFP-Rpn7 in *kap123null* cells, but not in *imp1null* cells.

Gene	Property	Localization		Importin α dependency	
		GFP-Rpn7	GFP-NLS		
sal3	Importin β	+	+	No	([13])
kap111	Importin β	+	+	ND	
kap123	Importin β	-	+	No	**([12], this study)**
kap114	Importin β	+	+	No	**([14, 15])**
kap104	Importin β	+	+	ND	
cut15	Importin α	+	ND	NA	
imp1	Importin α	+	-	NA	

Table 1, Localization of Rpn7 in importin α and β mutant cells.
Summary of Rpn7 localization in importin α and β mutant S. pombe cells thus far tested. "+"indicates normal localization, "-" indicates mislocalization, and ND indicates localization not determined. Localization of GFP-NLS is also listed as controls.